Fully Virulent *Bacillus anthracis* Does Not Require the Immunodominant Protein BclA for Pathogenesis[▽]

J. Bozue,† C. K. Cote,† K. L. Moody, and S. L. Welkos*

*Bacteriology Division, U.S. Army Medical Research Institute of Infectious Diseases,
1425 Porter Street, Fort Detrick, Maryland 21702*

The BclA protein is the immunodominant epitope on the surface of *Bacillus anthracis* spores; however, its roles in pathogenesis are unclear. We constructed a BclA deletion mutant (*bclA*) of the fully virulent Ames strain. This derivative retained full virulence in several small-animal models of infection despite the *bclA* deletion.

Bacillus anthracis is the causative agent of anthrax (6, 9, 18). The spore of *B. anthracis* is the infectious particle for all forms of the disease, including inhalational anthrax (6, 9, 18). There is significant support for the concept that spore antigens of *B. anthracis* contribute to protective immunity (1, 3, 5–7, 12, 15, 16, 28, 29).

The exosporium, the outermost spore structure, is a loose-fitting layer that envelops the spore (11). Approximately 20 exosporium-associated protein and glycoprotein species have been identified from analyses of *B. anthracis* and *Bacillus cereus* (2, 4, 21, 23–27). The exosporium membrane projects "hair-like" fibers (11), of which the major component is the BclA glycoprotein (23, 25). Upon identifying the immunodominant BclA protein, Sylvestre et al. observed no appreciable differences in virulence between the nonencapsulated toxinogenic Sterne vaccine strain and a *bclA* mutant derivative when spores were administered to mice subcutaneously (25). To further characterize the role of the BclA protein in *B. anthracis* pathogenicity, the *bclA* gene was deleted by allelic exchange and replaced with the Ω kan-2 fragment (20) from the chromosome of the fully virulent Ames strain of *B. anthracis*. The deletion was confirmed by PCR and Western blotting analyses, and as demonstrated by transmission electron and immunofluorescence microscopy, the Ames *bclA* mutant did not express the hair-like fibers projecting from the exosporium membrane present on wild-type Ames spores (data not shown).

We analyzed differences in the ability of spores of the *bclA* mutant to germinate, as it was previously shown that the exosporium contains enzymes that may affect spore germination (14, 21, 23, 27). To monitor both early and later germination events, we assayed the increase in fluorescence and decrease in absorbance of spores with times of incubation in media that induce germination (19, 30). We found no significant difference ($P > 0.05$) in germination between the strains by either assay (Fig. 1A and B).
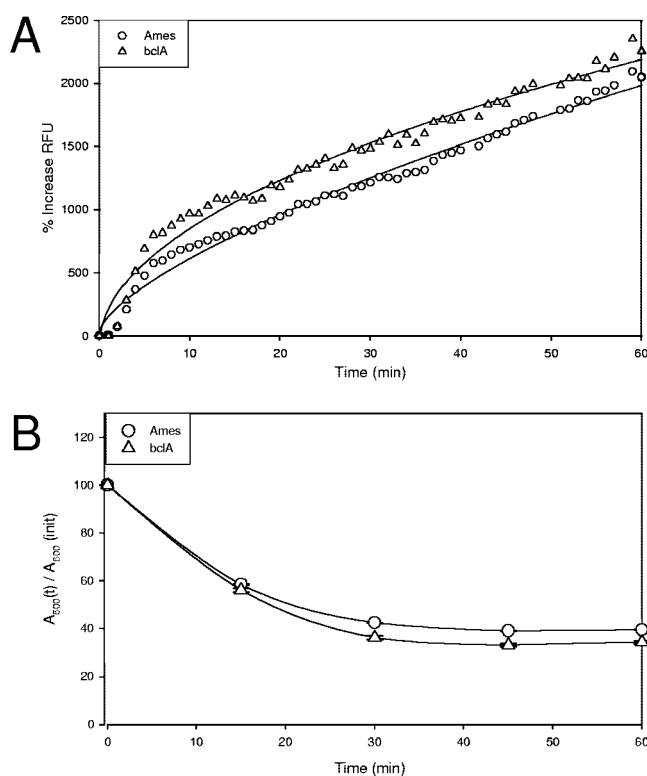

FIG. 1. Effect of the BclA protein on the germination rates of *B. anthracis* spores in vitro. (A) Microtiter spectrofluorometric assay of germination of spores in the presence of a germination medium with alanine, adenosine, and Casamino Acids (AAC), as described previously (30). The germination of spores of the wild-type Ames strain (○) and *bclA* strain (△) was monitored every minute for 1 h. The data (percent increase in relative fluorescence units [RFU]) are the relative increase in RFU at a given time point compared to the RFU at time zero, multiplied by 100. Representative data are presented, and similar results were obtained in at least two additional experiments. (B) Germination was also measured by absorbance readings as previously described (19). The A_{600} of each sample was measured at various times and is plotted as the percentage of the initial A_{600} [A_{600} (init)] at time zero that is represented by the A_{600} at a given time point [A_{600} (t)]. Absorbance data are from three independent experiments, and standard errors of the means are depicted.

* Corresponding author. Mailing address: Bacteriology Division, USAMRIID, 1425 Porter Street, Fort Detrick, MD 21702. Phone: (301) 619-4930. Fax: (301) 619-2152. E-mail: susan.welkos@amedd.army.mil.
† These authors contributed equally to this work.

Report Documentation Page

1. REPORT DATE **20 OCT 2006**	2. REPORT TYPE **N/A**	3. DATES COVERED **-**	
4. TITLE AND SUBTITLE **Fully virulent Bacillus anthracis does not require the immunodominant protein, BclA, for pathogenesis. Infection and Immunity 75:508 - 511.**		5a. CONTRACT NUMBER	
		5b. GRANT NUMBER	
		5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) **Bozue, J Cote, CK Moody, KL Welkos, SL**		5d. PROJECT NUMBER	
		5e. TASK NUMBER	
		5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD**		8. PERFORMING ORGANIZATION REPORT NUMBER **TR-06-092**	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)		10. SPONSOR/MONITOR'S ACRONYM(S)	
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT **Approved for public release, distribution unlimited**			
13. SUPPLEMENTARY NOTES			

14. ABSTRACT
The BclA protein is the immunodominant epitope on the surface of Bacillus anthracis spores; however, its roles in pathogenesis are unclear. We constructed a BclA deletion mutant (bclA) of the fully virulent Ames strain. This derivative retained full virulence in several small animal models of infection despite the bclA deletion.

15. SUBJECT TERMS
Bacillus anthracis, anthrax, spores, spore epitopes, Bc1A, dominance, characteristics, deletion mutant

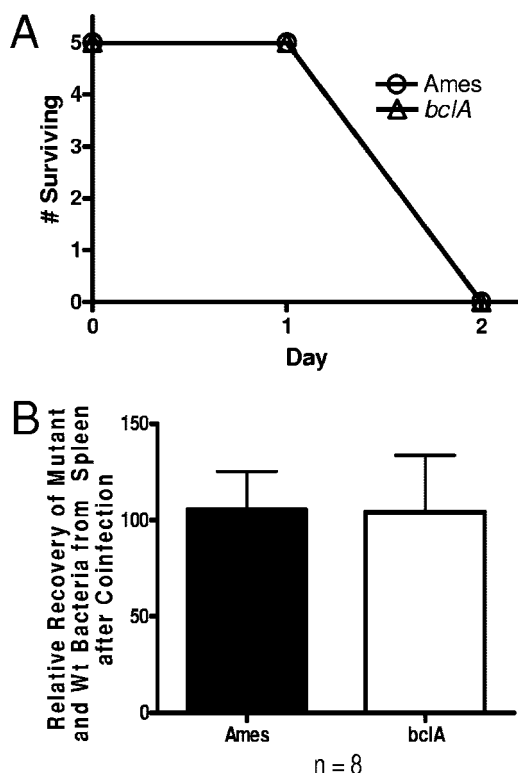| 16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**SAR**	**4**	

FIG. 2. Effect of the BclA protein on virulence in the guinea pig infection model. (A) Female Hartley guinea pigs were challenged i.m. with spores of the Ames wild-type strain (○) or spores of the *bclA* strain (Δ). (B) The effect of the *bclA* mutation on in vivo fitness in a competitive guinea pig infection model. Guinea pigs were coinfected i.m. with spores of both the Ames wild-type strain and the *bclA* strain. The error bars represent standard errors of the means. Wt, wild type.
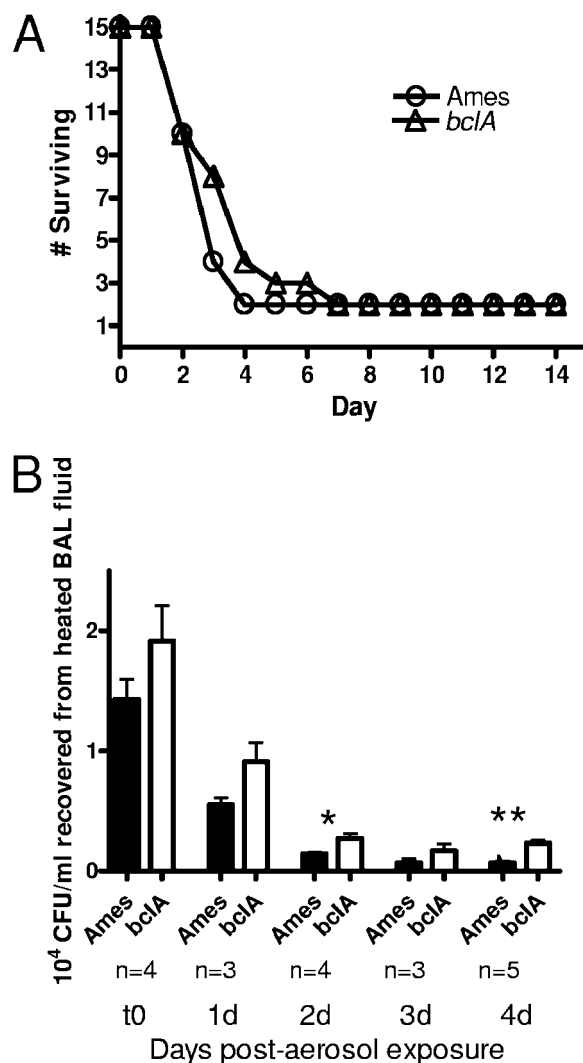

FIG. 3. Effect of the BclA protein on virulence in the mouse infection model. (A) BALB/c mice were challenged intranasally with spores of the Ames wild-type strain (○) or the *bclA* strain (Δ). (B) The effect of the *bclA* mutation on the rate of spore clearance from mouse lungs. Mice were exposed to an aerosolized inoculum of spores of both wild-type Ames and *bclA* mutant. The number of heat-resistant (65°C for 30 min) spores retained within the lungs at designated time points was determined by bacterial counts on selective and nonselective media. The error bars represent standard errors of the means. $*$, $P = 0.03$; $**$, $P = 0.0005$.

We examined the potential role of the BclA protein in pathogenesis using guinea pig intramuscular (i.m.) (13) and mouse intranasal (i.n.) (17) infection models. Guinea pigs were challenged i.m. with either approximately 980 spores of wild-type Ames or 860 spores of the *bclA* mutant. All guinea pigs succumbed to the infection by the second day after challenge (Fig. 2A). We also employed a more sensitive assay that utilizes in vivo competition in the guinea pig model. Guinea pigs were coinfected i.m. with approximately 1,000 spores. The spores delivered i.m. were an approximately equal mixture of wild-type Ames (57%) and *bclA* mutant (43%) spores. Two days later, moribund animals were euthanized and their spleens were harvested. Levels of bacterial load were determined within the spleens. Wild-type Ames and *bclA* bacteria were recovered from the spleen as measured by bacterial counts from Luria-Bertani (LB) agar plates (total number of bacteria) or LB agar plates containing kanamycin (number of *bclA* mutant bacteria only). Nearly identical relative percent recoveries were obtained when the recovered percent of splenic bacterial CFU was divided by the percent of spores in the challenge inoculum, suggesting that spores from both strains germinated, survived, and replicated at the same rate in vivo (Fig. 2B).

We also used a mouse model of i.n. infection in an attempt to identify differences in virulence associated with the *bclA*

mutation. BALB/c mice were challenged i.n. with either 9.25×10^5 spores of the Ames wild-type strain or 7.85×10^5 spores of the *bclA* mutant. There were no statistically significant differences observed in either survival rate or mean time to death between the two strains (Fig. 3A).

To further resolve the role of BclA in virulence, we developed a second in vivo competition assay to examine the rate of clearance of spores from the lungs of mice infected with aerosolized spores. Mice were exposed to an aerosol containing approximately equal concentrations of both the wild-type Ames (48%) and the *bclA* mutant (52%) spores; a calculated total inhaled dose of 4×10^5 spores was delivered (8). Mice were euthanized at various times postinfection, and bronchoal-

TABLE 1. Comparative rates of retention of the wild-type Ames and *bclA* mutant spores in the lungs of mice exposed to aerosol

Day	% Retention of strain[a]:	
	Ames	*bclA*
0	100	100
1	38.5 ± 4.1	47.4 ± 8.5
2	10.0 ± 1.0	14.2 ± 2.3
3	4.8 ± 2.5	8.8 ± 3.2
4	4.6 ± 0.9	12.1 ± 1.4

[a] Mean percent retention in BAL fluids (±standard errors of the means) was calculated as the percentage of the initial recovery at time zero.

veolar lavages (BAL) were performed. BAL fluid samples (both heated and unheated aliquots) were plated on LB agar plates or LB agar plates containing kanamycin. There were no differences noted between the results obtained from the heated (CFU counts representing heat-resistant ungerminated spores) (Fig. 3B) and unheated BAL fluid (data not shown), confirming that spore germination is negligible within the lungs themselves (8, 11a). Overall, these data suggested that both the wild-type Ames and the *bclA* mutant spores were cleared from the mouse lungs at similar rates (Fig. 3B; Table 1). However, on day 2 and day 4 there were statistically significant differences between the amounts of wild-type Ames and *bclA* mutant spores recovered from the BAL fluid (Fig. 3B). The implications of these findings for pathogenesis are unclear, as there were no significant differences observed at any other time point and the *bclA* mutant appeared to be of virulence equal to that of wild-type Ames by the pulmonary route (Fig. 3 and data not shown). However, these data suggest that the *bclA* mutant spores might be cleared slightly less efficiently from the lungs than wild-type spores, possibly because the mutant spores were able to bind better to host cells, such as the epithelial cells lining the lung and/or airway.

The exact roles of the BclA protein and the exosporium of *B. anthracis* in pathogenesis of and host protection against anthrax still remain to be determined. Several other species of *Bacillus* also possess an exosporium structure; however, except for *B. cereus*, these bacteria are not normally pathogens of mammals and are common environmental saprophytes. It was recently shown that spores of *B. anthracis* are able to germinate on and around roots of plants in a grass plant-soil model system (22). Perhaps the entire exosporium or the exosporial fibers are required for the interaction between spores and roots of the plants or serve in some other role for survival in the environment.

Studies such as the one reported here should contribute in several ways to the identification of novel vaccine candidates as well as targets for detector systems. First, a better understanding of how the immune response recognizes *B. anthracis* spores is necessary, particularly in relation to immunodominant antigens, such as BclA. More importantly, it is vital to characterize the roles of candidate spore antigens in bacterial pathogenesis and the role of the immune response in protective immunity. Our studies with a *bclA* mutant of the Ames strain revealed that BclA was not required for the pathogenesis of fully virulent *B. anthracis* in two animal species by different challenge methods. While BclA is immunodominant and accordingly an

attractive target for detection technologies, this protein could easily be removed from a *B. anthracis* variant engineered to evade detection systems, and yet these altered spores would retain full virulence.

REFERENCES

1. **Barnard, J. P., and A. M. Friedlander.** 1999. Vaccination against anthrax with attenuated recombinant strains of *Bacillus anthracis* that produce protective antigen. Infect. Immun. **67:**562–567.
2. **Bozue, J. A., N. Parthasarathy, L. R. Phillips, C. K. Cote, P. F. Fellows, I. Mendelson, A. Shafferman, and A. M. Friedlander.** 2005. Construction of a rhamnose mutation in *Bacillus anthracis* affects adherence to macrophages but not virulence in guinea pigs. Microb. Pathog. **38:**1–12.
3. **Brossier, F., M. Levy, and M. Mock.** 2002. Anthrax spores make an essential contribution to vaccine efficacy. Infect. Immun. **70:**661–664.
4. **Charlton, S., A. J. Moir, L. Baillie, and A. Moir.** 1999. Characterization of the exosporium of *Bacillus cereus*. J. Appl. Microbiol. **87:**241–245.
5. **Cohen, S., I. Mendelson, Z. Altboum, D. Kobiler, E. Elhanany, T. Bino, M. Leitner, I. Inbar, H. Rosenberg, Y. Gozes, R. Barak, M. Fisher, C. Kronman, B. Velan, and A. Shafferman.** 2000. Attenuated nontoxinogenic and nonencapsulated recombinant *Bacillus anthracis* spore vaccines protect against anthrax. Infect. Immun. **68:**4549–4558.
6. **Cote, C. K., D. J. Chabot, A. Scorpio, T. E. Blank, W. A. Day, S. L. Welkos, and J. A. Bozue.** 2006. *Bacillus anthracis*: agent of bioterror and disease, p. 83–111. *In* M. Bendinelli (ed.), Microorganisms and bioterrorism. Springer, New York, N.Y.
7. **Cote, C. K., C. A. Rossi, A. S. Kang, P. R. Morrow, J. S. Lee, and S. L. Welkos.** 2005. The detection of protective antigen (PA) associated with spores of *Bacillus anthracis* and the effects of anti-PA antibodies on spore germination and macrophage interactions. Microb. Pathog. **38:**209–225.
8. **Cote, C. K., N. van Rooijen, and S. L. Welkos.** 2006. The roles of macrophages and neutrophils in the early host response to *Bacillus anthracis* spores using a mouse model of infection. Infect. Immun. **74:**469–480.
9. **Friedlander, A. M.** 2000. Anthrax: clinical features, pathogenesis, and potential biological warfare threat. Curr. Clin. Top. Infect. Dis. **20:**335–349.
10. **Friedlander, A. M., S. L. Welkos, M. L. Pitt, J. W. Ezzell, P. L. Worsham, K. J. Rose, B. E. Ivins, J. R. Lowe, G. B. Howe, and P. Mikesell.** 1993. Postexposure prophylaxis against experimental inhalation anthrax. J. Infect. Dis. **167:**1239–1243.
11. **Gerhardt, P., and E. Ribi.** 1964. Ultrastructure of the exosporium enveloping spores of *Bacillus cereus*. J. Bacteriol. **88:**1774–1789.
11a.**Guidi-Rontani, C., M. Weber-Levy, E. Labruyere, and M. Mock.** 1999. Germination of *Bacillus anthracis* spores within alveolar macrophages. Mol. Microbiol. **31:**9–17.
12. **Hahn, U. K., R. Boehm, and W. Beyer.** 2006. DNA vaccination against anthrax in mice-combination of anti-spore and anti-toxin components. Vaccine **24:**4569–4571.
13. **Ivins, B. E., P. F. Fellows, and G. O. Nelson.** 1994. Efficacy of a standard human anthrax vaccine against *Bacillus anthracis* spore challenge in guinea-pigs. Vaccine **12:**872–874.
14. **Kang, T. J., M. J. Fenton, M. A. Weiner, S. Hibbs, S. Basu, L. Baillie, and A. S. Cross.** 2005. Murine macrophages kill the vegetative form of *Bacillus anthracis*. Infect. Immun. **73:**7495–7501.
15. **Kudva, I. T., R. W. Griffin, J. M. Garren, S. B. Calderwood, and M. John.** 2005. Identification of a protein subset of the anthrax spore immunome in humans immunized with the anthrax vaccine adsorbed preparation. Infect. Immun. **73:**5685–5696.
16. **Little, S. F., and G. B. Knudson.** 1986. Comparative efficacy of *Bacillus*

anthracis live spore vaccine and protective antigen vaccine against anthrax in the guinea pig. Infect. Immun. **52:**509–512.

17. **Lyons, C. R., J. Lovchik, J. Hutt, M. F. Lipscomb, E. Wang, S. Heninger, L. Berliba, and K. Garrison.** 2004. Murine model of pulmonary anthrax: kinetics of dissemination, histopathology, and mouse strain susceptibility. Infect. Immun. **72:**4801–4809.

18. **Mock, M., and A. Fouet.** 2001. Anthrax. Annu. Rev. Microbiol. **55:**647–671.

19. **Paidhungat, M., and P. Setlow.** 1999. Isolation and characterization of mutations in *Bacillus subtilis* that allow spore germination in the novel germinant D-alanine. J. Bacteriol. **181:**3341–3350.

20. **Perez-Casal, J., M. G. Caparon, and J. R. Scott.** 1991. Mry, a *trans*-acting positive regulator of the M protein gene of *Streptococcus pyogenes* with similarity to the receptor proteins of two-component regulatory systems. J. Bacteriol. **173:**2617–2624.

21. **Redmond, C., L. W. Baillie, S. Hibbs, A. J. Moir, and A. Moir.** 2004. Identification of proteins in the exosporium of *Bacillus anthracis*. Microbiology **150:**355–363.

22. **Saile, E., and T. M. Koehler.** 2006. *Bacillus anthracis* multiplication, persistence, and genetic exchange in the rhizosphere of grass plants. Appl. Environ. Microbiol. **72:**3168–3174.

23. **Steichen, C., P. Chen, J. F. Kearney, and C. L. Turnbough, Jr.** 2003. Identification of the immunodominant protein and other proteins of the *Bacillus anthracis* exosporium. J. Bacteriol. **185:**1903–1910.

24. **Steichen, C. T., J. F. Kearney, and C. L. Turnbough, Jr.** 2005. Character-

ization of the exosporium basal layer protein BxpB of *Bacillus anthracis*. J. Bacteriol. **187:**5868–5876.

25. **Sylvestre, P., E. Couture-Tosi, and M. Mock.** 2002. A collagen-like surface glycoprotein is a structural component of the *Bacillus anthracis* exosporium. Mol. Microbiol. **45:**169–178.

26. **Sylvestre, P., E. Couture-Tosi, and M. Mock.** 2005. Contribution of ExsFA and ExsFB proteins to the localization of BclA on the spore surface and to the stability of the *Bacillus anthracis* exosporium. J. Bacteriol. **187:**5122–5128.

27. **Todd, S., A. Moir, M. Johnson, and A. Moir.** 2003. Genes of *Bacillus cereus* and *Bacillus anthracis* encoding proteins of the exosporium. J. Bacteriol. **185:**3373–3378.

28. **Welkos, S., A. Friedlander, S. Weeks, S. Little, and I. Mendelson.** 2002. In-vitro characterisation of the phagocytosis and fate of anthrax spores in macrophages and the effects of anti-PA antibody. J. Med. Microbiol. **51:**821–831.

29. **Welkos, S., S. Little, A. Friedlander, D. Fritz, and P. Fellows.** 2001. The role of antibodies to *Bacillus anthracis* and anthrax toxin components in inhibiting the early stages of infection by anthrax spores. Microbiology **147:**1677–1685.

30. **Welkos, S. L., C. K. Cote, K. M. Rea, and P. H. Gibbs.** 2004. A microtiter fluorometric assay to detect the germination of *Bacillus anthracis* spores and the germination inhibitory effects of antibodies. J. Microbiol. Methods **56:**253–265.

Editor: J. L. Flynn